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Expression of insulin-like growth factor system genes in liver and brain tissue during embryonic and post-hatch development of the turkey

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Abstract

A molecular cloning strategy employing primer-directed reverse transcription polymerase chain reaction (RT-PCR) was devised to sequence 1300 bp of a turkey liver-derived cDNA corresponding to the complete coding region and the 5′- and 3′-untranslated regions of the insulin-like growth factor (IGF)-II mRNA transcript (GenBank accession no. AF074980). The turkey IGF-II gene codes for a 187 amino acid precursor protein that includes a signal peptide, the mature IGF-II hormone, and a C-terminal extension peptide comprised of 24, 67 and 96 amino acids, respectively. Turkey IGF-II showed greater than 95% sequence identity at both the nucleotide and amino acid level with chicken IGF-II. Expression of IGF-I, IGF-II, IGF type-I receptor (IGF-IR), and IGF binding protein (IGFBP)-2 and -5 genes was quantified relative to an internal 18S rRNA standard by RT-PCR in liver and whole brain tissue on days 14, 16, 18, 20, 22, 24 and 26 of embryonic development, as well as at hatch (H, day 28) and at 3 weeks post-hatching (PH). Expression of liver IGF-I was low throughout embryonic development, but increased more than 8-fold by 3 weeks PH. In contrast, IGF-I was expressed in brain tissue at much higher levels than liver throughout development and this level of expression in brain increased gradually, reaching its highest point at 3 weeks PH. IGF-II was expressed at comparable levels in brain and liver tissue during embryonic development, except for transient increases in liver just prior to hatching (days 24 and 26) and at 3 weeks PH. Expression of IGF-IR declined in brain throughout development reaching its lowest level at 3 weeks PH. In liver, IGF-IR expression was lower than that of brain throughout development. An inverse relationship was observed for the expression of IGF-I and IGF-IR genes in brain, but not in liver, through 3 weeks PH. Expression of the IGFBP-2 gene increased in liver around the time of hatch (days 26–28) and declined by 3 weeks PH, whereas the level of expression of IGFBP-5, which was higher than IGFBP-2, remained fairly constant in both brain and liver throughout the developmental period studied. Our data indicates differential expression of selected genes that comprise the IGF system in the turkey during embryonic and PH growth and development.

Keywords: Development; Gene expression; Growth; Insulin-like growth factor system; IGF-I; IGF-II; IGF-1R; IGFBP; Turkey

1. Introduction

The insulin-like growth factor (IGF) system, consisting of two growth factors (IGF-I and IGF-II), two IGF receptors (IGF-1R and IGF-2R/cation-independent manose 6-phosphate receptor, M6P-R) and six IGF binding proteins (IGFBP1-6), has been characterized as an important regulatory system for controlling tissue growth and development in vertebrate species (Jones and Clemmons, 1995). Both IGF-I and IGF-II are mitogenic peptides which play important roles in regulating cellular proliferation, differentiation, and metabolism in different tissues (Stewart and Rotwein, 1996). The IGFBPs have been reported to regulate IGF ligand activity by influencing their bioavailability to IGF receptors (Clemmons, 1998). In birds, IGF-I and IGF-II act as endocrine and/or as paracrine/autocrine signals whose physiological effects are mediated mainly by their binding to a common receptor (IGF type I receptor, IGF-1R) and partly through the insulin receptor (McMurtry et al., 1997). An IGF-2R (M6P-R) has been previously identified and characterized in chickens that does not to bind IGF-II and,

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therefore, lacks this important functionality characteristic of its mammalian counterpart (Canfield and Kornfeld, 1989; Duclos and Goddard, 1990; Yang et al., 1991; Zhou et al., 1995). In birds, both IGF peptide ligands (I and II) and their common receptor (IGF-IR) are structurally similar to insulin and the insulin receptor, respectively (McMurtry et al., 1997). In mammals, IGF-I is thought to play a pivotal role in mediating the effects of growth hormone (GH) on tissue growth during postnatal development, whereas IGF-II has been reported to function primarily during embryonic growth and development (Dupont and Holzenberger, 2003). The liver is considered to be the main source of circulating IGF-I and it has been shown that IGF-I in plasma and extracellular fluids is bound, specifically and with high affinity, to IGFBPs that regulate its bioavailability and thereby modulate the growth-promoting effects of IGF-I (Dupont and Holzenberger, 2003).

Expression of IGF system genes has been studied throughout development of mammals in many different tissues including liver, muscle, bone, reproductive organs, and the central nervous system (Allan et al., 2001; Dupont and Holzenberger, 2003). The IGF system, however, has not been studied as intensively in birds as it has been in mammals (McMurtry et al., 1997). There have been a number of reports concerning the effects of development and nutritional status on expression of IGF-I, IGF-II, IGF-IR, IGFBP2, and IGFBP5 genes in different avian tissues (McMurtry et al., 1997; McMurtry, 1998; Allan et al., 2001). Also, there have been several reports on developmental changes in circulating levels of the IGFs and IGFBPs in developing chicken and turkey embryos (McMurtry et al., 1996, 1997, 1998). Although some avian IGF system genes have been identified and characterized including IGF-I, IGF-II, IGF-IR, M6P-R, IGFBP2, and IGFBP5, with the exception of IGF-I (GenBank accession no. AF074980) sequence information has not yet been reported for any of these genes for the turkey. Therefore, our goals in this study were: 1) to identify and characterize the turkey IGF-II gene homologue and 2) to investigate changes in the level of expression for IGF system genes in liver and brain tissues during embryonic and post-hatch development of the domestic turkey (*Meleagris gallopavo*).

2. Materials and methods

2.1. Animals

Fertile turkey (*M. gallopavo*) eggs were incubated in a standard commercial incubator. On incubation days 14, 16, 18, 20, 22, 24, 26, and 28 (hatching), 5 eggs were removed and samples of liver and brain tissue were collected. Turkey poults were reared from hatching to 3 weeks of age in heated battery/brooder units. They received a standard starter poultry ration and water ad libitum. Samples of liver and brain tissue were collected at 3 weeks of age. All tissue samples were snap frozen in liquid nitrogen and stored at −80 °C prior to RNA isolation. Turkey genomic DNA was extracted from 50 μL of whole blood collected from newly hatched poults using the Easy DNA extraction kit (Invitrogen/Life Technologies, Carlsbad, CA). All protocols involving the use of animals received prior approval from the Beltsville Animal Care and Use Committee.

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen/Life Technologies). Reverse transcription (RT) reactions (20 μL) consisted: of 1.0 μg total RNA, 50 units Superscript II reverse transcriptase (Invitrogen/Life Technologies), 40 units of an RNAse inhibitor (Invitrogen/Life Technologies), 0.5 mM dNTPs, and 100 ng random hexamer primers. Polymerase Chain Reaction (PCR) was performed in 25 μL reactions containing: 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.0 unit of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs, 2.0 mM Mg^{2+}, 10 pmol of each gene specific primer (Table 1), 5 pmol each of

Table 1
Gene-specific primers employed for analysis of turkey IGF system genes by relative quantitative RT-PCR

Gene	Accession number	Primer sequence (5′→3′)	Orientation	Product size (bp)
1. IGF-I	AF074980	GCTGAGCTGGTTGATGCTCT	Forward	202
		CACGTACAGAGCGTGCAGAT	Reverse	
2. IGF-II	–[a]	GGGACAGGGGCTTCTACTTC	Forward	205
		GGCTTCTGGAAGCTCTCCTT	Reverse	
3. IGF-1 Receptor	AJ223164[b]	CAAGCATGCGTGAGAGGATA	Forward	404
		CAAACCTTTCCCTCCTTTCC	Reverse	
4. IGFBP-2	GGU15086[b]	AACAGGCATGAAGGAGATGC	Forward	400
		CTGCTCATGGGCTGTGTAGA	Reverse	
5. IGFBP-5	–[c]	CCTCTCTGACCTCAAAGCTGA	Forward	198
		ACCATCCTCTGGCTGCTCT	Reverse	

[a] Primer set designed from derived sequence (this study) for turkey IGF-II.
[b] Chicken sequences were used to design these primer sets since the corresponding turkey sequence was lacking.
[c] Primer set designed from chicken EST sequence for IGFBP-5 (EST #038617.1; Boardman et al., 2002). This primer set also shows 100% identity with the corresponding sequence in Japanese quail IGFBP-5 gene (GenBank accession no. AF293839).

an appropriate mixture of primers:competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion, Inc., Austin, TX), and 1 μL of the RT reaction. Thermal cycling parameters were 1 cycle 94 °C for 2 min, followed by 30 cycles, 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 8 min.

2.3. Nucleotide sequencing

Liver and brain total RNA and primer-directed RT-PCR were used to generate turkey IGF-II cDNAs that were sequenced. Primer sets based on sequence reported for zebra finch and chicken (GenBank accession nos. AJ223165, AY043225, respectively) were initially used to generate a series of overlapping PCR products. Gene-specific PCR primers were designed to span projected exon/intron junctions for sequencing genomic DNA. PCR products were subjected to bi-directional automated fluorescent DNA sequencing utilizing a Beckman Coulter CEQ 8000XL Genetic Analysis System using a dye terminator cycle sequencing kit (Beckman Coulter, Inc., Fullerton, CA, USA). PCR products were evaluated by agarose gel (1.5%) electrophoresis and bands of the appropriate size were excised from the gel and purified using a GenElute gel extraction kit (Sigma-Aldrich, St. Louis, MO, USA) or purified directly from the PCR sample by a GenElute PCR clean-up kit (Sigma).

2.4. Rapid amplification of cDNA ends (RACE)

Liver and brain poly A^+ selected RNA (1.0 μg) were used to prepare 3′- and 5′-RACE ready cDNA using the SMART RACE cDNA Amplification Kit (BD Biosciences/Clontech, Palo Alto, CA, USA). PCR was performed using Platinum Taq DNA polymerase with 3.5 mM Mg^{2+} (Invitrogen), touchdown PCR, and gene specific 3′- and 5′-RACE primers.

2.5. Cloning

Cloning of selected PCR products containing both the 5′- and 3′-untranslated regions (UTRs) of turkey IGF-II gene was performed using a TOPO TA Cloning kit with a pCR 2.1 TOPO vector (Invitrogen). The inserted DNA was sequenced using M13 forward and reverse primers supplied with the kit.

2.6. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF)

Isolation and quantification of PCR products was accomplished using CE-LIF as described previously (Richards and Poch, 2002). Briefly, aliquots (2 μL) of RT-PCR samples were first diluted 1:100 with deionized water. A P/ACE MDQ (Beckman Coulter, Inc.) equipped with an argon ion LIF detector was used. Capillaries were 75 μm I.D. × 32 cm μSIL-DNA (Agilent Technologies, Palo Alto, CA, USA). EnhanCE™ dye (Beckman) was added to the DNA separation buffer (Sigma) to a final concentration of 0.5 μg/mL. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 5 min. P/ACE MDQ software (Beckman) was used to calculate peak areas for the PCR products separated by CE.

2.7. Determination of gene expression by relative RT-PCR

The levels of IGF-I, IGF-II, IGF-IR, IGFBP-2, and IGFBP-5 gene expression in liver and brain tissues were determined as the ratio of integrated peak area for each IGF system gene PCR product relative to that of the co-amplified 18S rRNA internal standard (Ambion, Inc.). Values are presented as the mean ± S.E.M. of 5 individual expression ratio determinations.

2.8. Statistical analyses

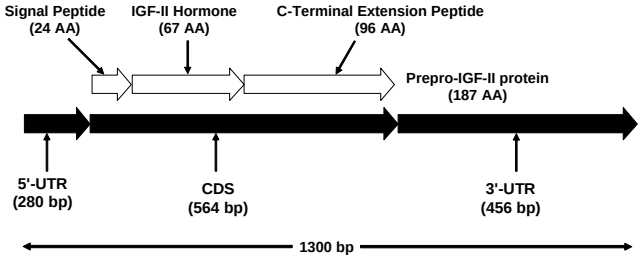
The effect of developmental age (days) on gene expression levels in liver and brain tissue was analyzed by two-way ANOVA (with developmental age and tissue as the main effects) using the general linear models (GLM) procedure of SAS software (The SAS System for Windows, v. 8.2; SAS Institute Cary, NC, USA). Differences among individual means were evaluated using the Duncan's multiple range test option of the GLM procedure for SAS software. Significance for mean differences was set at $P < 0.05$. Linear regression was used to relate developmental changes in the expression of IGF-I, IGF-II and IGF-IR gene expression in brain.

3. Results

3.1. Molecular cloning of the turkey IGF-II cDNA and preliminary characterization of gene structure

A turkey IGF-II gene homologue has now been identified and characterized (GenBank accession no. AF074980). A cDNA derived from IGF-II mRNA was sequenced yielding 1300 bp that encompassed the complete coding region and the 5′- and 3′-untranslated regions (Fig. 1). From this information we conclude that the turkey IGF-II mRNA encodes a 187 amino acid precursor protein that displays greater than 95% homology with chicken IGF-II at both the nucleotide and amino acid level and 60–70% homology with mammalian IGF-II sequences (Tables 2 and 3). Zebra finch IGF-II displayed an intermediate level of homology, closer to chicken than mammalian forms. The highest degree of homology across species was observed for that region of the IGF-II gene that encodes the mature IGF-II hormone, whereas lesser homologies were observed for the regions encoding the signal peptide and the C-terminal peptide (data not shown).

Preliminary mapping of the turkey IGF-II gene indicated a structure similar to the chicken gene (Darling



Feature	Location (nt)	Location(aa)
5' Untranslated Region	1-280	-
Coding Region	281-844	1-187
Signal Peptide	281-352	1-24
Pro IGF-II	353-841	25-187
IGF-II	353-553	25-91
C-Terminal Extension Peptide	554-841	92-187
3' Untranslated Region	845-1300	-
Poly A Signal	1292-1297	-

Nucleotide and Translated Amino Acid Sequences[1,2,3]

```
   1  GCGCTCGCCT GCTCTCTAGA ATAACAAAAT ACCTGATCGT AACAAAATAA CATCCTCCAG ACCAAATCAA GCAGGGACTT GACATACCAA AAGGCACAGC
 101  GAGGAGAGCG AGCCAGGGCG CGGGGAAGGC GAGCCAGAGC CGCGATCGCT GCCAGGGCCG GACAGCAGCA TGGCAAGCGC CGGGGCACAC ACGGATGAGC
   1                                                                                          M  C  A  A  R  Q  M
 201  GCTGCCGGCA GCCTGCCTTC CTCCCCGGCC CGCCGCCGAC AGAAGTTGAG AGCGGCAGCG GCAGCGCCAA GGTGCAGAGG ATGTGTGCAG CCAGGCAGAT
   8    L  L  L  L  L  A  F  L  A  Y  A  L  D  S  A  A  A  Y  G  T  A  E  T  L  C  G  G  E  L  V  D  T  L
 301  GCTGCTGCTA CTGCTGGCCT TCCTGGCCTA TGCATTGGAT TCGGCTGCAG CGTATGGCAC GGCAGAGACC CTCTGTGGTG GGGAGCTGGT GGACACACTG
  41    Q  F  V  C  G  D  R  G  F  Y  F  S  R  P  V  G  R  N  N  R  R  I  N  R  G  I  V  E  E  C  C  F  R
 401  CAGTTCGTCT GTGGGGACAG GGGCTTCTAC TTCAGTAGAC CAGTGGGACG AAATAACAGG AGGATCAACC GTGGTATCGT GGAGGAGTGC TGCTTTCGGA
  74    S  C  D  L  A  L  L  E  T  Y  C  A  K  S  V  K  S  E  R  D  L  S  A  T  S  L  A  G  L  P  A  L  N  K
 501  GCTGCGACCT GGCTCTGCTG GAAACCTACT GTGCCAAGTC CGTCAAGTCA GAGCGTGACC TCTCCGCCAC CTCCCTTGCG GGCCTCCCAG CCCTCAACAA
 108    E  S  F  Q  K  P  S  H  A  K  Y  S  K  Y  N  V  W  Q  K  K  S  S  Q  R  L  Q  R  E  V  P  G  I  L
 601  GGAGAGCTTC CAGAAGCCAT CTCACGCCAA GTACTCCAAG TACAACGTGT GGCAGAAGAA GAGCTCGCAG CGGCTGCAGC GGGAGGTGCC TGGCATCCTG
 141    R  A  R  R  Y  R  W  Q  A  E  G  L  Q  A  A  E  E  A  R  A  M  H  R  P  L  I  S  L  P  S  Q  R  P
 701  CGTGCCCGCC GGTACCGGTG GCAGGCAGAG GGGCTGCAAG CAGCCGAGGA AGCCAGGGCG ATGCATCGTC CCCTCATCTC CTTGCCCAGC CAGCGGCCCC
 174  P  A  P  R  A  S  P  E  A  T  G  P  Q  E  *
 801  CGGCGCCGCG GGCATCCCCT GAAGCGACCG GCCCCCAGGA ATGAACTGTG ACCGGCCGGC TCGATTTGTG ATCTCCCGGG GAGAGACTGG CGAGACTCGG
 901  CCCCCCCTGA GCCCCTCCGT CCCCAAGCCG AGGAGCGGGG CGGCAGGCAC CATCACGCCG CTCCGGCCCC AACCAAACAA CTGACACAAG CAAGGAGGGG
1001  ATGGCAGCGG CCGTGTGCAG CGTCCTCCTT TTGGGGTGGG GGGGAGGGTT GTTTCTTTTC CCAGGCCTGT TTCGTTTCCC TCGCGCACCC AGTATTTTTC
1101  AAGCCTTGTA CTTACGAAGG GACAGTTGGG CACTTGAACT TTTTGCTGCC GGGCGCTGTG TGACATCCCC AGTGCCAACA CTAGAAGACA AAAGAACGAG
1201  CAAGAGGGAA AAAGGATATG AAGTTAACTT TTCTTTTAAA TTTTATTTTA TTTTTCTCAA GAAGAAGAGT CCATTCCGTG TCTTTCTTGA CAATAAAGTG
                                                                                                    Poly A Signal
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[1] Coding region (CDS) nucleotide sequence is highlighted by underlining. Translated amino acid sequence is shown above in single letter code.

[2] Translated amino acid sequence of the mature IGF-II hormone is highlighted by shaded box.

[3] Numbering to the left denotes nucleotide number (regular font) and translated amino acid number (bold italic font).

Fig. 1. Graphic representation and nucleotide and amino acid (AA) sequence for the turkey IGF-II cDNA/mRNA (GenBank accession no. AY829236). The locations and size (in bp or AA) of the untranslated regions (3′- and 5′-UTR), coding region (CDS), signal peptide, mature IGF-II hormone, and C-terminal extension peptide are indicated. The translation of the complete CDS is shown in single letter AA code above the corresponding nucleotide sequence.

and Brickell, 1996) including 4 exons (3 of which contained the entire coding region) and 3 introns (Fig. 2). Using turkey genomic DNA as a template, we sequenced a portion of the IGF-II gene corresponding to the third intron, which was found to be 713 bp in size (GenBank accession no. AY829237). Interestingly, the nucleotide sequence for intron 3 was found to share >90% identity with the corresponding region of the chicken IGF-II gene (739 bp), indicating a high degree of gene sequence homology among these two avian species extending beyond the coding region (data not shown).

3.2. Expression of IGF system genes in liver and brain tissue during embryonic and post-hatch development of the turkey

Throughout embryonic development, IGF-I gene expression in liver was significantly lower than in brain tissue. By

Table 2
Species comparison of insulin-like growth factor-II open reading frame

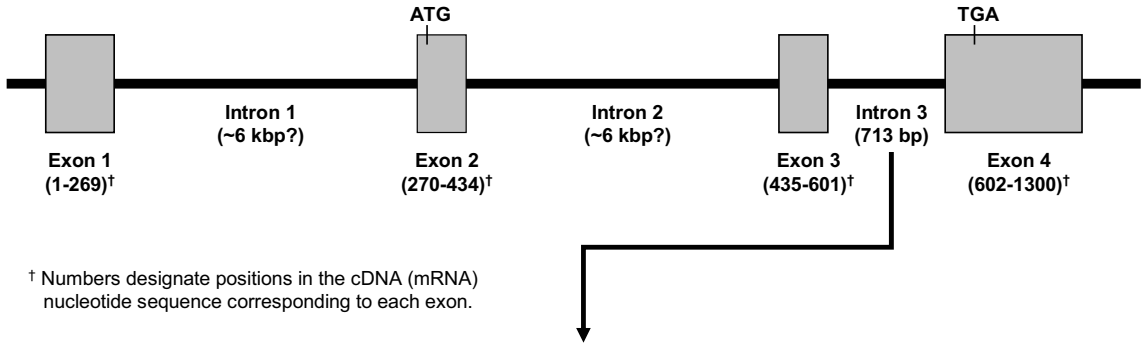
	Size (bp)	Human	Pig	Mouse	Zebra finch	Chicken	Turkey
Human[1]	543	100	89	87	66	64	64
Pig[2]	546		100	84	68	64	65
Mouse[3]	543			100	64	62	62
Zebra Finch[4]	564				100	87	88
Chicken[5]	564					100	96
Turkey	564						100

Percentage of nucleotide identities.
GenBank accession nos. [1]AF517226, [2]X56094, [3]NM010514, [4]AJ223165, [5]AH005039.

Table 3
Species comparison of insulin-like growth factor-II open reading frame

	Size (aa)	Human	Pig	Mouse	Zebra finch	Chicken	Turkey
Human[1]	180	100	88(91)	83(89)	57(69)	58(71)	58(71)
Pig[2]	181		100	79(85)	58(70)	58(71)	58(71)
Mouse[3]	180			100	58(69)	59(70)	59(70)
Zebra finch[4]	187				100	90(95)	90(95)
Chicken[5]	187					100	99(100)
Turkey	187						100

Percentage of amino acid identities and conservative changes.
GenBank accession nos. [1]AAM51825, [2]CAA39574, [3]NP034644, [4]CAA11145, [5]AAB46818.

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  1   cggagctgcg acctggctct gctggaaacc tactgtgcca agtccgtcaa gtcagagcgt
 61   gacctctccg ccacctccct tgcgggcctc ccagccctca acaagGTAGG GCTGCACTCG
121   GGCTGCTAGC TCCCTGAAGC AAGAAAGAGG AGAAAAGGGA GGCCATCAGT AGGGGAGGAC
181   TGCTGCTGGC TCTACGATCT GCTGTTTCTG CAGCCAAGGC AGCATGCAGA GCCTTGAAGT
241   GTTTATAACT GTTGAGGAGT CAAAGAAATT GCAGAAAACA AACTTCTAAC CATTGACTCA
301   GAACGGTTTG GGTTGGAAGA GCCCCCTAGT GATTATCTAG TCCACCTCTC CGCCATGGGC
361   AGGGACACAT CCCACTCTTA GATCAGATTG CCCGAAGGCC CATCCAGCCT GGTCTTGAAT
421   GCCTTCAGAG AGAGGGCACC CACAGCTCCT CTGGACAGTC TGTCCCAGCA TCTTTGTCCC
481   ACCCTGTTGT GGAAAACTTC CTCCTTACGT GCAGTTTCCT TCAGCTTAAA ATCATTGCCC
541   CTTGGCCTAT CCCTACAGGC TCCGGTAAAA AGTTTTTCTC CAGCTGCAGC AAGGTCTCCC
601   TGGAACCTTT TCCAGGCTCA ACAACCCCAA CTCTCCCCAC CTTTCCTCAT AGGAGAGGTA
661   TTTCCAGCTT GCTAATAATT CTTGTGGCTT TCCTCCGTCC ACATCCTCCT TGCGCTGGGG
721   ACCCAATAGA ACCTGTAACA TGGGCAACCG TGGGTAGAGA TTACCCCAGG GAAGGTAACC
781   CACCTTGTGT TGAGACTGCG TTGTTCTCCC TTCCCCAGga gagcttccag aagccatc
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Fig. 2. A proposed structure for the turkey IGF-II gene. The positioning and size (in bp) of each of the 4 exons and 3 introns is indicated. The positions of the start codon (ATG, located in exon 2) and stop codon (TGA, located in exon 4) are also indicated. Intron 3 nucleotide sequence (106–818) derived from genomic DNA templates is designated by uppercase letters. The surrounding lowercase letters correspond to flanking nucleotide sequence from exons 3 (1–105) and 4 (819–838).

3 weeks PH, IGF-I gene expression was significantly ($P<0.05$) elevated above the level at H (day 28) in both liver and brain. The increase in liver IGF-I gene expression from hatch to 3 weeks PH was dramatic representing more than 8-fold increase which is similar in magnitude to the difference in IGF-I expression in brain as compared to liver during embryonic development (Fig. 3).

Expression of IGF-II increased significantly ($P<0.05$) relative to 18S rRNA in liver just prior to hatch (days 24 and 26) and again at 3 weeks PH (Fig. 4). Expression of IGF-II in brain tissue exhibited a gradual decline starting on day 20 of embryonic development and continuing to 3 weeks PH, although this change was not significant ($P>0.05$). Up until day 22 of incubation, IGF-II expression in brain exceeded that of liver, but this difference was not as dramatic as that observed for IGF-I expression.

In liver, IGF-1R gene expression was variable. However, the expression of this gene was consistently and significantly ($P<0.05$) higher in brain as compared to liver throughout development with a decline starting on

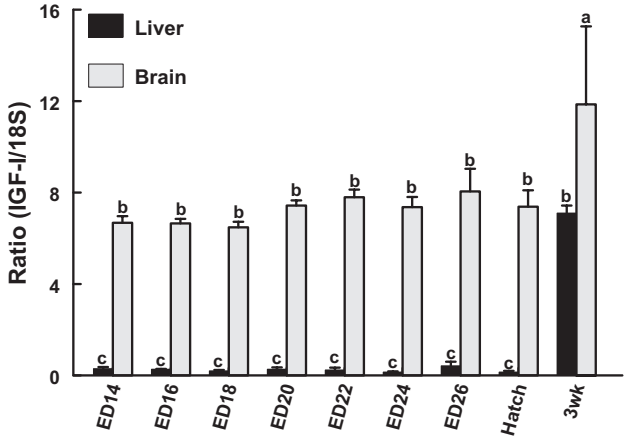

Fig. 3. Expression of the IGF-I gene in turkey brain and liver tissue during embryonic development (days 14–26), at hatch (day 28) and at 3 weeks post hatch. RT-PCR was used to quantify the level of expression relative to an 18S rRNA internal standard. Values represent the mean±S.E.M. of 5 determinations. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons.

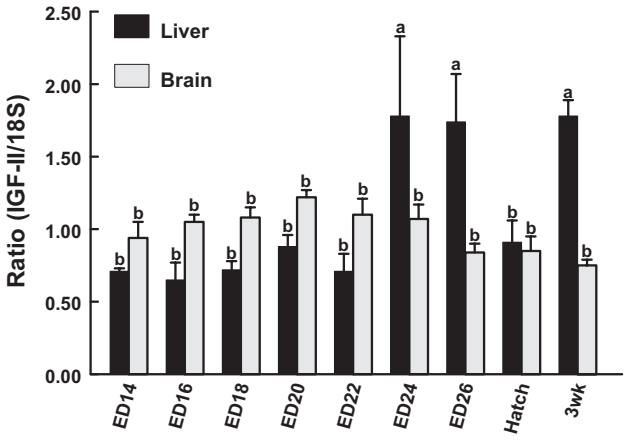

Fig. 4. Expression of the IGF-II gene in turkey brain and liver tissue during embryonic development (days 14–26), at hatch (day 28) and at 3 weeks post hatch. RT-PCR was used to quantify the level of expression relative to an 18S rRNA internal standard. Values represent the mean±S.E.M. of 5 determinations. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons.

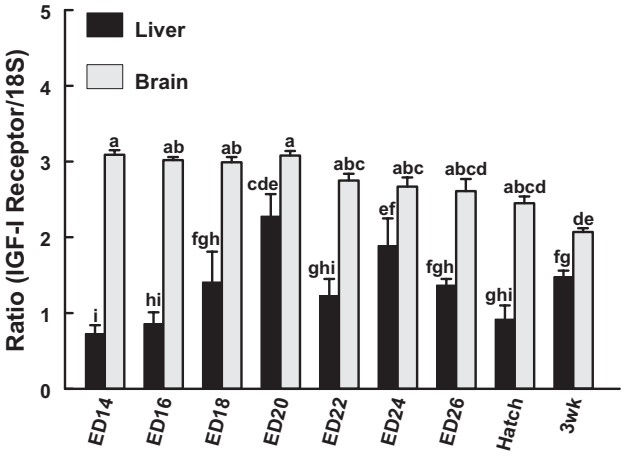

Fig. 5. Expression of the IGF-I receptor gene in turkey brain and liver tissue during embryonic development (days 14–26), at hatch (day 28) and at 3 weeks post hatch. RT-PCR was used to quantify the level of expression relative to an 18S rRNA internal standard. Values represent the mean± S.E.M. of 5 determinations. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons.

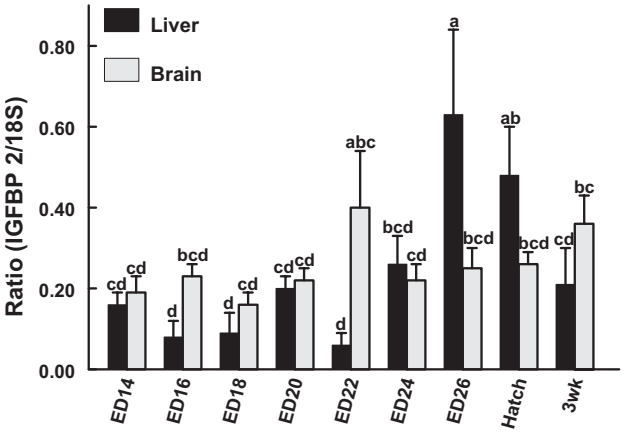

Fig. 7. Expression of the IGFBP-2 gene in turkey brain and liver tissue during embryonic development (days 14–26), at hatch (day 28) and at 3 weeks post hatch. RT-PCR was used to quantify the level of expression relative to an 18S rRNA internal standard. Values represent the mean± S.E.M. of 5 determinations. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons.

day 20 of embryonic development and continuing to 3 weeks PH (Fig. 5).

Linear regression analysis of IGF-I, IGF-II and IGF-1R gene expression data, during brain tissue development, indicated an inverse correlation ($r^2=-0.712$) between the transcriptional activity of IGF-I and IGF-1R genes. In contrast, expression of the IGF-II gene showed a positive correlation ($r^2=0.579$) with the expression of IGF-IR. These correlations were observed in brain but not in liver tissue. Taken together, these findings suggested the possibility of coordinate regulation of IGF-I, IGF-II, and IGF-IR gene expression in brain tissue from late embryonic development through 3 weeks PH (Fig. 6).

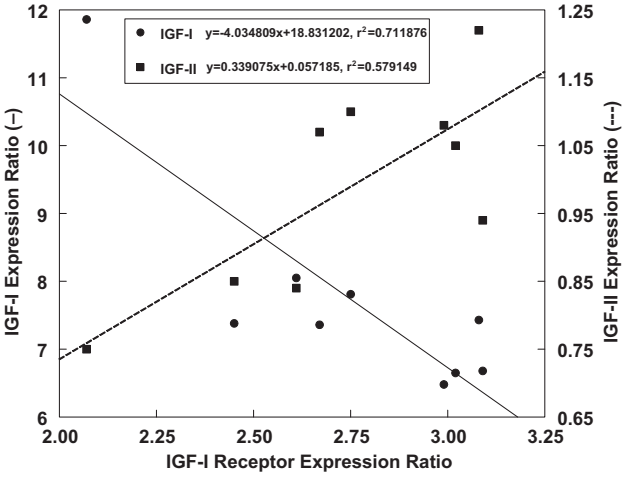

Fig. 6. Mean expression ratios (relative to 18S rRNA) of IGF-I and IGF-II plotted against the mean expression ratio for IGF-I receptor in brain throughout development. Linear regression analysis was used to characterize the relationship between the expression of the two IGF genes and the receptor. The equations used to plot the lines and the coefficients of determination (r^2) for IGF-I and IGF-II are shown. Each point represents the mean of 5 determinations for each gene.

Expression of the IGFBP-2 gene was low in both liver and brain tissue throughout the course of this study, during both embryonic and PH development (Fig. 7). There was a significant ($P<0.05$) increase in hepatic IGFBP-2 expression peaking just prior to hatching (day 26) followed by a decline through 3 weeks PH. Similarly, expression of IGFBP-2 in brain tissue tended to increase as development proceeded, though the trend was not significant ($P>0.05$). Expression of IGFBP-5 was much higher in both liver and brain than observed for IGFBP-2 (Fig. 8). On days 14–18, on day 22, and at 3 weeks PH expression of IGFBP-5 was significantly ($P<0.05$) higher in brain than it was in liver. However, no major expression trends were observed in either tissue for this IGFBP.

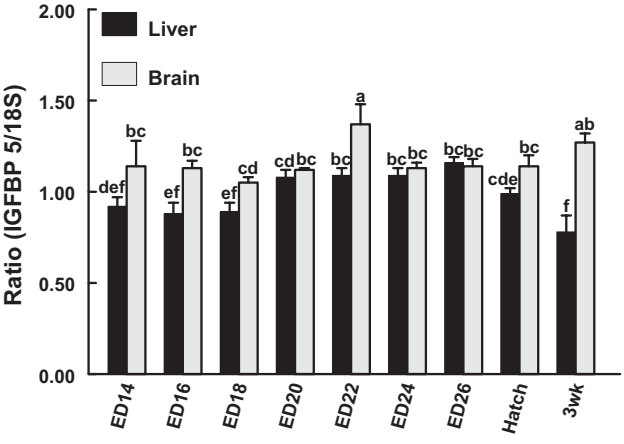

Fig. 8. Expression of the IGFBP-5 gene in turkey brain and liver tissue during embryonic development (days 14–26), at hatch (day 28) and at 3 weeks post hatch. RT-PCR was used to quantify the level of expression relative to an 18S rRNA internal standard. Values represent the mean± S.E.M. of 5 determinations. Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons.

4. Discussion

4.1. Nucleotide sequence and structure of the turkey IGF-II mRNA transcript and gene

Using molecular cloning techniques, we have identified and sequenced a portion of the turkey IGF-II gene corresponding to the entire coding region and the 5′- and 3′-untranslated regions (UTRs) of the mRNA transcript. The turkey gene encodes a putative precursor protein consisting of a 24 amino acid signal peptide, a 67 amino acid mature IGF-II peptide hormone, and a 96 amino acid C-terminal peptide. Turkey IGF-II demonstrated high homology (>95%) with chicken IGF-II at both the amino acid and nucleotide levels. Furthermore, the predicted turkey IGF-II peptide (67 amino acids) displayed 100% amino acid identity when compared with its chicken counterpart. Although a cDNA has been identified and sequenced for the turkey IGF-I gene (GenBank accession no. AF074980), this is the first report to characterize the sequence and structure of a turkey IGF-II gene homologue. There is currently very limited information concerning the nucleotide sequence, gene structure or expression for other members of the turkey IGF system (Ernst et al., 1996), although there have been a number of reports concerning detection of IGF-I, IGF-II, IGF-1R and IGFBPs at the protein level (McFarland et al., 1992, 1993; Sun et al., 1992; McMurtry et al., 1994, 1996, 1997, 1998; McMurtry and Brocht, 1997; Halevy et al., 2003). An IGF-II gene homologue has also been identified and characterized in the zebra finch (*Taenopygia guttata*) and a comparison of the coding region demonstrated 88% and 94% nucleotide and amino acid sequence identity, respectively with turkey IGF-II (Holzenberger et al., 1997). Like the chicken (Darling and Brickell, 1996), the turkey IGF-II gene is comprised of four exons, three of which contain the entire coding region (564 bp), and three introns. The third intron of the turkey IGF-II gene determined in this study is interspersed within the region that codes for the C-terminal peptide portion of the IGF-II protein precursor and it displayed a high degree of nucleotide sequence homology (>90%) with the corresponding region in the chicken IGF-II gene (Darling and Brickell, 1996).

From Northern analysis, IGF-II mRNA transcript size has been estimated to range from 1.4 to 8.2 kb in chickens (Holzenberger and Lapointe, 2000) and 0.9 to 4.0 kb in the zebra finch (Holzenberger et al., 1997). The 1.4 kb size is similar to the cDNA clone reported here as well as cDNAs reported for zebra finch (GenBank accession no. AJ223165) and quail (Holzenberger et al., 1997). However, it has also been suggested that the smaller transcripts (e.g., 1.4 kb) may not be efficiently polyadenylated and that a 4.0 kb transcript is the one that is enriched in poly A^+ selected RNA (Holzenberger et al., 1997). Alternatively, Holzenberger and Lapointe (2000) reported that a 2.7 kb transcript was the predominant mRNA species detected by Northern analysis of chicken brain total RNA samples, and they suggested the possibility of tissue-specific expression of IGF-II mRNA transcripts that vary in size. The presence of a poly A signal motif at the 3′ end of the turkey transcript does indicate the potential for polyadenylation to occur on the 1.3 kb turkey IGF-II transcript. However, the reported heterogeneity in avian IGF-II transcript size could arise from differing lengths of the 3′-UTR generated by the use of alternative polyadenylation sites. Thus, progressively larger mRNA transcripts could be derived from extension of the transcript at its 3′ end. Such a scenario is supported by an examination of the 3′-flanking sequence of the chicken IGF-II gene contained on chromosome 5 (GenBank accession no. AP003796) which does contain multiple downstream polyadenylation signal motifs including one that could give rise to a transcript of 3.97 kb. Moreover, it is now recognized that the 3′ region of gene transcripts can influence their level of expression (Kakoki et al., 2004), and thus, this may represent a potential mode of regulation for IGF-II expression in different tissues or at different times during development. Multiple IGF-II mRNA transcripts have been reported previously in rats with differences in relative abundance of each in different tissues and subject to developmental regulation (Graham et al., 1986; Nielsen, 1992). Clearly, this situation remains to be explored for the turkey IGF-II gene. However, it is important to note that a single IGF-II mRNA transcript of approximately 4.0 kb was reported using Northern analysis of total RNA isolated from turkey myogenic satellite cells and a labeled rat cDNA probe (Ernst et al., 1996).

In the chicken, the IGF-II gene is present on chromosome 5. With the availability of flanking sequence from the chicken genome and the observed high degree of sequence identity outside of the coding region exhibited between chicken and turkey, it should be possible to utilize the same primer-directed (‘primer walking’) PCR strategy utilized in this study to clone additional portions of the turkey IGF-II gene beyond those reported here. These could include 5′-flanking sequence encompassing the promoter region as well as other portions such as the two large (~6 kb) and, still uncharacterized, introns (1 and 2, see Fig. 2), and the 3′-flanking region. Thus, a comparative genomics approach should be useful in developing a more complete understanding of the structure and regulation of the turkey IGF-II gene. Moreover, a similar strategy may prove to be useful for a more in-depth study of other turkey IGF system genes that, as of yet, remain to be fully characterized.

4.2. Expression of IGF-I and IGF-II genes in liver and brain tissue during development

In liver, expression of the IGF-I gene was low throughout embryonic development before increasing dramatically (~8 fold) by 3 weeks PH. This is consistent with several observations concerning the regulation of the hepatic IGF-I gene. First, it has been previously noted in mammalian

species that liver-derived IGF-I in circulation plays an important role during postnatal development in mediating the growth-promoting effects of GH (Dupont and Holzenberger, 2003). However, there have been reports indicating that circulating levels of GH and IGF-I are not correlated during embryonic development in chickens (Johnson et al., 1990; Kikuchi et al., 1991). Burnside and Cogburn (1992) reported that IGF-I mRNA levels increased rapidly in the liver of chickens, reaching their highest level at 4 weeks PH similar to a peak in plasma GH at 3–4 weeks PH. After this period, they concluded that some elements of the GH/IGF-I regulatory pathway were not tightly coordinated during late (10 weeks) PH development of the chicken. Throughout incubation of the turkey, plasma levels of IGF-I were found to be lower than IGF-II, whereas by 3 weeks PH, both growth factors reached a similar level in circulation (McMurtry et al., 1998). This may reflect a role for the liver in determining circulating levels of both growth factors during embryonic development in turkeys. However, the expression of IGF-I and IGF-II by chicken embryonic liver is apparently not influenced by changes in circulating GH which is detectable only during late incubation and PH periods (McMurtry et al., 1998). Furthermore, expression of IGF-I in extra-hepatic tissues, such as brain, was found to be independent of the influence of GH in chickens before and after hatching, whereas IGF-I mRNA was expressed in liver in a GH-dependent manner only after hatching (Rosselot et al., 1995; Tanaka et al., 1996).

There have been no previous descriptions of IGF-II expression in turkey tissues other than a single report of a decline in IGF-II mRNA levels during differentiation of satellite cells cultured from postnatal breast muscle (Ernst et al., 1996). McMurtry et al. (1998) were the first to describe developmental changes in plasma IGF-II in turkeys. Elevated levels of circulating IGF-II and high levels of hepatic and brain gene expression prior to hatching are consistent with an important role for this growth factor during embryonic development of the turkey as has been suggested previously for the chicken (McMurtry et al., 1997; Holzenberger and Lapointe, 2000). It is interesting to note that a dramatic rise in circulating IGF-II during embryonic development, peaking at 26 days, followed by a dramatic decline at hatch has been reported for the developing turkey (McMurtry et al., 1998). This is similar to hepatic expression of IGF-II mRNA observed in this study and could indicate a role for the liver in determining circulating levels of IGF-II during embryonic development of the turkey. The importance of IGF-II to avian embryogenesis has been suggested to involve its role in cellular proliferation, cartilage growth, as well as, in the uptake of glucose and amino acids (McMurtry et al., 1997). In general, expression of IGF-II is greater than IGF-I in tissues throughout embryonic development in birds which is consistent with findings in other species that IGF-II is the significant IGF during embryonic development (Stewart and Rotwein, 1996; Allan et al., 2001). However, it does not appear that IGF-II regulates growth after hatching in chickens (Spencer et al., 1996).

4.3. Expression of IGF-IR in liver and brain tissue during development

The IGF-1R, which exhibits tyrosine kinase activity, plays a key role throughout development in birds by regulating the growth and differentiation of a variety of tissues including those of the central nervous system (Holzenberger et al., 1996). Brain tissue has been reported to express the highest levels of IGF-1R mRNA compared to liver and other tissues during embryonic development of chickens (Armstrong and Hogg, 1994, Holzenberger et al., 1996). Our findings suggest that a similar situation may exist for the turkey during embryonic development. Significant expression of the IGF-1R gene in both liver and brain throughout turkey embryonic and PH development suggests an important role of IGF hormones in the development of these two tissues. Although, IGF-1R protein has been detected in turkey liver and brain tissues, it is most likely that turkeys, like chickens, lack a functioning type II IGF receptor (manose-6-phosphate receptor) based on ligand blotting experiments using ^{125}I-labeled IGF-I and -II (McFarland et al., 1992; Sun et al., 1992). Furthermore, the existence of a type II receptor gene has yet to be demonstrated in the turkey as has been found for chickens (Canfield and Kornfeld, 1989; Duclos and Goddard, 1990; Yang et al., 1991; Zhou et al., 1995). Therefore, IGF-1R would be expected to mediate transmembrane signal transduction for both IGF-I and IGF-II in turkeys as it apparently does in the chicken (McMurtry et al., 1997). The high level of expression of IGF-1R in both liver and brain during embryonic development and continuing on into the PH period in this study is consistent with that role. Moreover, Holzenberger and Lapointe (2000) demonstrated a significant amount of overlap in the expression of IGF-1R and that of IGF-I and IGF-II by in situ hybridization and immunocytochemical analyses suggesting paracrine/autocrine signaling by the IGF system in the brain during later stages of chicken embryogenesis and possible coordinate expression of the IGF hormones and their type I receptor.

The inverse (IGF-I) and positive (IGF-II) relationships found in this study to exist between the expression of both IGF genes and the type-I receptor gene could indicate a possible coordinated developmental regulation for these IGF system genes in turkey brain tissue. LeRoith et al. (1995) reported that physiological conditions such as fasting (during which IGF-I levels in circulation decline) upregulated expression of the IGF-1R gene in rats, whereas IGF-I was found to decrease IGF-1R mRNA levels when administered to cultured cells. These observations are consistent with the inverse relationship observed in this study between the expression of IGF-I and IGF-1R genes during development of the turkey brain. Because hepatic expression of IGF-I was found to be so low during

embryonic development of the turkey, it was not possible to accurately determine if an inverse relationship existed for the expression of these two genes in liver. However, such a relationship could develop during the PH period in turkeys when IGF-I gene expression rises significantly. Armstrong and Hogg (1992, 1994) reported that liver IGF-1R mRNA levels declined during the first 4 weeks PH in chickens, a time during which hepatic expression of IGF-1 increases. We have previously reported an inverse relationship in the expression of IGF-I and IGF-1R genes in skeletal muscle tissue from 3-week-old broiler chickens in response to a 48 h fast (Evock-Clover et al., 2002). Matsumura et al. (1996) reported that a 5-day fast of 4-week-old chickens increased liver IGF-1R mRNA by about 2-fold but the level in brain failed to change. They concluded that development- and tissue-specific modes of regulation were involved in IGF-1R gene expression in response to changes in nutritional status of young growing chickens. However, Ernst et al. (1996) found no evidence for autocrine regulation by IGF-II of IGF-1R expression in turkey muscle satellite cells as had been previously suggested for BC_3H-1 mouse myoblast cells by Rosenthal et al. (1991). Thus, it remains unclear what specific factor(s) might influence the expression of IGF-1R in different turkey tissues and clearly more work is required to understand the regulation of this gene in avian species.

4.4. Expression of IGFBP-2 and IGFBP-5 genes in liver and brain tissue during development

Six distinct IGFBP genes have been cloned and sequenced for mammalian species. These have been subsequently shown to be potent modulators (both negative and positive) of IGF actions on cells and tissues and they may also exhibit ligand-independent activities (Jones and Clemmons, 1995; Allan et al., 2001; Dupont and Holzenberger, 2003). However, to date, only IGFBP-2 and -5 genes have been identified and characterized in chickens (Schoen et al., 1995; Allander et al., 1997). Although it has been reported that both turkey (McMurtry et al., 1996) and chicken (Francis et al., 1990; Beccavin et al., 1999) plasma contain specific IGFBPs of different sizes that bind IGF-I and IGF-II, it appears that the main (~30 kDa) IGFBP present in chicken plasma is IGFBP-2 (McMurtry et al., 1997; Radecki et al., 1997; Beccavin et al., 1999). Kita et al. (2002) suggested that IGFBP-2 serves as a negative regulator of postnatal growth in chickens by modulating the growth-promoting effects of IGF-I in response to different nutritional states. Our findings indicated that expression of IGFBP-2 in turkey liver and brain tissue was low throughout development. Ernst et al. (1996) reported that IGFBP-2 mRNA levels were highest in proliferating myogenic satellite cells derived from turkey breast muscle and this level decreased significantly as differentiation progressed. Radecki et al. (1997) reported that circulating levels of IGF-II and IGFBP-2 exhibited similar patterns during the peri-hatch period and suggested that IGFBP-2 may serve as a "sink" for circulating IGF-II to bind this hormone and affect its biological activities during this time. They went on to suggest that the events of hatching, but not elevated corticosterone levels in blood, could be involved in increased expression of IGF-II and IGFBP-2 during the peri-hatching period. Our observations that hepatic expression of IGF-II and IGFBP-2 exhibited similar patterns with a peak in expression just prior to hatching (see Figs. 4 and 7) suggests that these two genes may be regulated in a coordinate fashion in the developing turkey. However, it is not known which tissue(s) contribute to the high circulating levels of IGFBP-2 observed in birds.

In mammals all of the IGFBPs, including IGFBP-5, are found in the circulation (Rajaram et al., 1997; Baxter et al., 2002). However, to date, there have been no reports of specific detection of IGFBP-5 or the association of IGF ligands with this binding protein in the blood of birds. This is in contrast to several reports of mRNA expression of IGFBP-5 in different avian tissues (Allander et al., 1997; Onagbesan et al., 1999; Fu et al., 2001; Allan et al., 2003; Heck et al., 2003). Similarly, in this study we found a high level of IGFBP-5 gene expression in both liver and brain tissue throughout embryonic and early PH development of the turkey. Moreover, this is the first report of IGFBP-5 gene expression in the turkey. In mammals IGFBP-5 is present in circulation at a low concentration relative to other more abundant binding proteins (IGFBP-2 and -3). Also, IGFs bound to IGFBP-5 are readily sequestered by the extracellular matrix indicating that this particular IGFBP may play a role in storage of IGF in extracellular spaces to be released for subsequent utilization in tissue growth or remodeling (Rajaram et al., 1997; Dupont and Holzenberger, 2003). This finding could help to explain the apparent inability to detect IGFBP-5 in avian circulation and may represent a potential mode of action for this particular IGFBP as part of the paracrine/autocrine regulation of IGF system-mediated cell growth and metabolism in the turkey. Furthermore, Allan et al. (2003) reported that expression of the IGFBP-5 gene during early embryogenesis in chickens is subject to regulation by sonic hedgehog, a key factor involved in patterning of the neural tube, somites and limbs, and that this may be important in establishing body symmetry in the avian embryo.

4.5. Conclusions

Our data suggests a potential role for the IGF system in regulating tissue growth and development of the turkey as has been found for other vertebrate species. We have identified and characterized a turkey IGF-II gene homologue as well as explored changes in the expression of other genes of the IGF system family. We conclude that differential expression of IGF system genes occurs in the turkey during embryonic and post-hatch growth and development.

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